DEOXYRIBONUCLEASE IIβ PROTEINS AND cDNAs

INTRODUCTION

This invention was made in the course of research sponsored by the National Institutes of Health. The U.S. Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Controlled cell death is critical for the life of a human; too much cell death can cause the symptoms of cystic fibrosis and also lead to diseases such a neurodegeneration and acquired immune deficiency syndrome (AIDS). In contrast, too little cell death can lead to cancer or autoimmune diseases. Recent studies have defined the pathway of cell death as "apoptosis" and have identified some of the biochemical steps involved.

Apoptosis is a homeostatic mechanism involved in the controlled death of obsolete cells during metamorphosis, differentiation, cell turnover, and hormone mediated deletion of thymocytes (Wyllie et al. *Int. Rev. Cytol.* 1980 **68**:251-306). Apoptosis has also been identified as the mechanism of cell killing during growth factor withdrawal (Rodriguez-Tarduchy et al. *EMBO J.* 1990 **9**:2997-3002; McConkey et al. *J. Biol. Chem.* 1990 **265**:3009-3011), T-cell deletion, treatment with many cytotoxic agents (Cohen, J.J. and Duke, R.C. *J. Immunol.* 1984 **132**:38-42; Barry et al. *Biochem. Pharmacol.* 1990 **40**:2353-2362; Kaufmann, S.H. *Cancer Res.* 1989 **49**:5870-5878; and McConkey et al. *Science* 1988 **242**:256-259), and following hyperthermia (Barry et al. *Biochem. Pharmacol.* 1990 **40**:2353-2362; Lennon et al. *Biochem. Soc. Trans.* 1990 **18**:343-345; Takano et al. *J. Pathol.* 1991 **163**:329-336).

Central to the mechanism of apoptosis is internucleosomal DNA digestion by endogenous endonucleases. Mammalian cells contain a variety of endonucleases which could be involved in internucleosomal DNA digestion. It was originally postulated that the primary endonuclease involved in apoptosis is a Ca^{2+}/Mg^{2+}-dependent endonuclease. Several Ca^{2+}/Mg^{2+}-dependent endonucleases have been identified, one of which is deoxyribonuclease I (DNase I), (Peitsch et al. *EMBO J.* 1993 **12**:371).

Recent experiments, however, indicate that DNase I may not be the primary endonuclease involved in apoptosis. It has been found that many cells do not contain this endonuclease. The role of DNase I, or any other Ca^{2+}/Mg^{2+}-dependent endonuclease is further unlikely, as often no increase or only a minor increase in Ca^{2+} levels occurs in apoptotic cells (Eastman, A. *Cell Death and Differentiation* 1994 **1**:7-9).

An alternate endonuclease that is active below pH 7.0 and has no apparent requirement for Ca^{2+} or Mg^{2+} has been detected (Sorenson et al., *J. Natl Cancer Inst.* 1990 **82**:749). This alternate endonuclease was identified as deoxyribonuclease II (DNase II; Barry, M.A. and Eastman, A. *Archives of Biochem and Biophys.* 1993 **300**(1):440-450). It was proposed that this enzyme is involved in the internucleosomal digestion or fragmentation of DNA which is one of the early steps in the pathway of apoptosis. Another report that has implicated DNase II in cell death involves lens fiber cell differentiation, a process where the cells lose their nuclei in a manner similar to apoptosis (Torriglia, A. et al. 1995 J. Biol. Chem. 270:28579-28585). In this process, the chromatin condenses and the cells degrade their genomic DNA. DNase II was found by immunocytochemistry to be localized in the cytoplasm but translocated to the nucleus of the fiber cell before degeneration. These findings implicate DNase II as the endonuclease responsible for genomic degradation

observed during lens nuclear degeneration, and further support a role for this enzyme in mechanisms of cell death.

However, more recent results have implicated yet another endonuclease, referred to CAD or caspase-activated deoxyribonuclease, in apoptosis (Enari, M. et al. 1998 Nature 391:43-50). Thus, it remains to be determined which specific endonuclease is involved in apoptosis,

The enzyme referred to herein as deoxyribonuclease IIα (DNA IIα) was isolated and purified and the amino acid sequence determined (PCT/US97/18262). The DNA sequences for both the human and bovine proteins of DNase IIα have also been cloned (PCT/US97/18262). Use of DNA IIα in alleviating the suffering in patients with cystic fibrosis is also disclosed in this PCT application.

In cystic fibrosis, the lungs of patients fill with the remnants of dead cells, and in particular with the DNA from these dead cells. The presence of DNA makes the mucous plugs too viscous to expel. A suggested therapy for these symptoms is the use of DNase I to digest the DNA, thereby permitting expulsion of the mucous plugs. However, this therapy has not been particularly effective due to inactivity of the DNase I enzyme in the presence of actin, also present in the sputum.

It is believed that DNase II enzymes and variations thereof may provide a more effective therapeutic alternative.

Another isoform of the DNase II enzyme, referred to herein as deoxyribonuclease IIβ (DNase IIβ) has now been identified and the gene and protein sequences for the mouse and human homolog have been determined.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a cDNA encoding deoxyribonuclease IIβ.

Another object of the present invention is to provide an isolated, purified deoxyribonuclease IIβ enzyme.

Yet another object of the present invention is to provide antibodies against this protein which can be used in diagnosing cells at various stages in the apoptotic pathway.

Yet another object of the present invention is to provide antisense agents targeted to a cDNA or corresponding mRNA encoding deoxyribonuclease IIβ.

Yet another object of the present invention is to provide a method for identifying agents that inhibit DNase IIβ activity comprising treating cells with a test agent, transfecting cells with DNase IIβ, maintaining said transfected cells in culture, and monitoring apoptosis in treated and untreated cells to determine whether the test agent modulates apoptosis.

Yet another object of the present invention is to provide a method for inducing apoptosis in selected cells comprising transfecting cells with a vector expressing the DNase IIβ cDNA so that apoptosis is induced.

Yet another object of the present invention is to provide a method of digesting DNA released from dead cells with an effective amount of an isolated, purified DNase IIβ protein so that DNA is digested.

DETAILED DESCRIPTION OF THE INVENTION

The existence of a deoxyribonuclease II (DNase II) enzyme as a protein of lysosomal origin that is involved in cellular digestion of foreign DNA has been known for many years. Recently, a DNase II enzyme has been linked with the DNA fragmentation that occurs at an early stage in apoptosis. The bovine and human forms of this DNase II protein, referred to herein as DNase IIα protein have been isolated and purified and the amino acid sequences of these proteins are disclosed in PCT/US97/18262. cDNAs encoding the bovine and human form of DNase IIα have also been cloned and characterized in PCT/US97/18262.

An isoform of this enzyme, referred to herein as deoxyribonuclease IIβ (DNase IIβ) has now been identified.

This full length gene for this isoform was first identified in mice by sequence comparison to expressed sequence tags entered in Genbank database which were similar, but not identical to DNase IIα. Oligonucleotide primers were synthesized to obtain the complete DNase IIβ mouse gene. The mouse DNase IIβ cDNA sequence is depicted as SEQ ID NO:1. The protein sequence of mouse DNase IIβ is depicted in SEQ ID NO:2.

Information from the mouse sequence was used to isolate a human homolog of this gene. The human DNase IIβ cDNA sequence is depicted as SEQ ID NO:3. The protein sequence of human DNase IIβ encoded by the cDNA of SEQ ID NO:3 is depicted in SEQ ID NO:4.

Mouse and rat cDNAs of this homolog of DNase IIα have also been disclosed recently by Shiokawa and Tanuma (*Nucleic Acid Res.* 1999 **27**(20):4083-4089 and *Biochemical and Biophysical Research Communications* 1999 **285**:395-399).

It has been found that the DNase IIβ protein, like the DNase IIα protein, retains a critical histidine in the predicted active site thus indicating that these proteins have similar activities. However, there is sufficient difference in the region surrounding this histidine to suggest that their activities, and in particular their potential as a therapeutic for cystic fibrosis, may be slightly different. Specifically, the predicted active site of human DNase IIα is FNSTEDHSKWCV (SEQ ID NO:5) while the equivalent sequence in the human DNase IIβ isoform is FSSYQDHAKWCI (SEQ ID NO:6).

Further, it has now been found that DNase IIβ is expressed at high levels in human salivary glands and is secreted into the saliva.

Using fluorescence *in situ* hybridization (FISH), it has now been determined that the human DNase IIβ is located at

chromosome 1p22. Chromosome 1p22 is frequently a lost or rearranged region in numerous types of cancer including breast, lymphoma, liver and mesothelioma. While several genes in this region have been investigated, no clear candidate for the tumor suppressor at this locus has been identified. DNase IIα is lethal when reintroduced into cells. Based on sequence similarity, it is expected that its isomer DNase IIβ will have similar activity. Since this cell killing activity is consistent with the function of tumor suppressor genes, it is believed that DNase IIβ could represent the tumor suppressor that is lost in these types of tumors. Accordingly, the mouse and human DNase IIβ gene sequence and protein of the present invention are believed to be useful in the development of assays, screening approaches and targeted therapies for cancer.

For example, polymerase chain reaction (PCR) techniques can be used to determine whether the gene is missing or mutated in cancer cells. Such cells are expected to be more susceptible to the introduction of foreign genes through means such as gene therapy.

Identification of agents which increase DNase IIβ expression are expected to be useful in suppressing tumor formation and/or inducing apoptosis in cells. Inducing apoptosis is not only useful in treatment of cancer, but also in the treatment of various autoimmune disorders such as multiple sclerosis in which immune cells that recognize the normal patient tissue have failed to die as should normally happen.

The mouse and human DNase IIβ gene sequence and protein of the present invention are also useful in the development of agents which decrease expression of endogenous DNase IIβ in cells. For example, antisense agents targeted to a portion of the cDNA sequence of the present invention or the corresponding mRNA can be developed. These antisense agents can then be used to decrease or inhibit the expression of

DNase IIβ thereby protect cells from premature death. These antisense agents may therefore be useful in treating diseases resulting from too much cell death such as neurodegeneration and AIDS.

Accordingly, cDNAs of the present invention are useful in identifying agents which modulate, i.e., increase or decrease, apoptosis in cells. In this method, cells from a single culture are divided in two groups. The first group, referred to as the treated cells, are placed in contact with a test agent in a vehicle. The second group, referred to as untreated cells, are placed in contact with vehicle only. Treated and untreated cells are then transfected with the cDNA of the present invention and apoptosis in the treated and untreated cells is monitored to determine whether treating cells with the test agent modulates apoptosis in the cells.

In addition, the DNase IIβ proteins of the present invention or fragments thereof are useful as antigens to produce antibodies thereto. By "antibody" it is meant to include, but is not limited to, both polyclonal and monoclonal antibodies as well as chimeric, single chain, and humanized antibodies along with Fab fragments, or the product of a Fab expression library. Various techniques for producing such antibodies are well known in the art.

Polyclonal antibodies generated against DNase IIβ can be obtained by direct injection of the isolated, purified proteins of the present invention or fragments thereof into an animal, preferably a nonhuman. Such antibodies can then be used to isolate the enzyme from tissues expressing that enzyme.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Such techniques are used routinely by those skilled in the art. Some examples include, but are not limited to, the hybridoma technique, the trioma technique, the

human B-cell hybridoma technique and the EBV-hybridoma technique.

These antibodies are useful in studying the expression of DNase IIβ in a variety of cells. DNase IIβ levels can be determined in selected cells by contacting selected cells with the antibody against DNase IIβ and detecting binding of antibody to deoxyribonuclease IIβ enzyme in the selected cells. For example, in one embodiment, an antibody of the present invention is used to detect the intact protein in normal human cells compared to tumor cells to determine whether the tumor cells fail to express the endonuclease.

DNase IIα digests DNA. Thus, given the similarity between DNase IIα and the IIβ isoform of the present invention, it is believed that DNase IIβ will also digest DNA. Patients suffering from cystic fibrosis have viscous sputum in their lungs; accumulation of this viscous sputum can lead to suffocation. Much of this viscosity comes from the release of DNA from cells dying in the lungs. DNase I is currently used in patients with cystic fibrosis as an inhaler to digest DNA in the mucous plugs of the lungs of these patients. However, this enzyme is inhibited by actin, also present in sputum. Thus, the efficacy of this treatment is limited. Previously, DNase II enzymes would not have been considered a practical alternative because enzymatic activity was only observed at a pH below that of the lungs. However, the low pH activity of DNase IIα is associated with a small DNase II fragment rather than the full length protein. The full length DNA IIα and DNA IIβ identified herein may have other catalytic activities such as an ability to digest DNA at higher pH. Accordingly, it is believed that administration of a concentration of a DNase II enzyme which causes digestion of DNA in sputum will be effective in alleviating suffering of patients with cystic fibrosis by decreasing the viscosity of the sputum in the lungs.

The following nonlimiting examples are provided to further illustrate the present invention.

Examples

Example 1: Identification of expressed sequence tags

The cDNA sequence of DNase IIα was submitted to the Genbank database on a regular basis for analysis against the rapidly accumulating data deposited therein to identify other cDNA and protein sequences with similarity to DNase IIα. An expressed sequence tag (EST) from mouse cDNA was identified that has high similarity to DNase IIα. These EST sequences are random pieces of cDNA that have been partially sequence but have no known function. The identified mouse EST was purchased and completely sequenced. This sequencing revealed a complete cDNA sequence with considerable homology to DNase IIα, but with sufficient differences that it obviously represented a different gene.

Additional EST sequences from human tissues were found that had similarity to this mouse EST. However, upon sequencing they contained incomplete sequences. Specifically, EST # AI420898, whose sequence was deposited into Genbank on March 28, 1999 was found to contain 932 bp of the gene referred to herein now as DNase IIβ. This sequence was cloned into pT7T3D-Pac vector from Pharmacia.

Example 2: Nucleic Acid Sequencing

Plasmid DNA obtained in Example 1 was sequenced using the Big-DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems, followed by analysis on an Applied Biosystems 370 DNA automated sequencer.

Example 3: Genomic Localization

Human genomic DNA was used as a substrate for PCR using oligonucleotide primers predicted from the homology with DNase

IIα to span intron 5 of DNase IIβ. A 2,000 base pair fragment was isolated and cloned into the PCR-script vector. This genomic fragment was biotinylated and used as a probe in fluorescent in situ hybridization to whole chromosomes. The probe hybridized to chromosome 1p22.